

SUMMARY

A convenient method is described for obtaining the genetic variants of α_s -caseins in high purity. Subsequent to chemical fractionation, the α_s -caseins were chromatographed on DEAE-cellulose in the presence of 3.3 M urea. α_s -A, B, and C caseins contain 1.01% phosphorus and 15.10, 15.34, and 15.40% nitrogen, respectively. Each is stabilized against precipitation with Ca^{++} by κ -casein. Due to the presence of other α_s -like caseins in cow's milk, the authors have proposed that the genetic variants of the fraction called α_s -casein be termed α_{s1} -A, α_{s1} -B, and α_{s1} -C. Further identification of the α_{s1} -caseins was made by their relative electrophoretic mobilities on starch-gel and polyacrylamide-gel.

Caseins from the milks of individual cows are genetically variable in the α_s - (5, 14, 15) and β -casein fractions (1). Three electrophoretic forms of each component, differing only slightly in mobility, have been detected by starch-gel electrophoresis (SGE) and polyacrylamide-gel electrophoresis (PAE). Papers I (5) and II (13) of this series were concerned with the mode of inheritance of the polymorphs of α_s - and β -caseins.

For a casein component to qualify as an α_s -casein, by the definition of Waugh et al. (19), certain conditions must be met. First, the component must interact with κ -casein at appropriate stoichiometric weight ratios to form ultracentrifugal complexes. Second, in the presence of κ -casein, the component must be capable of forming stable micelles clottable upon the addition of rennin. This definition of α_s -casein is, of course, an operational definition which seems satisfactory, at least for the present. Among the questions yet to be answered concerning this definition is—What is the appropriate weight ratio for the formation of α_s - κ complexes?

The purpose of this paper is to report the isolation, characterization, and some properties of α_{s1} -caseins A, B, and C in relation to similar fractions described by others. To avoid

confusion in interpreting results of this study, reasons for our change in nomenclature from α_s -A, B, and C to α_{s1} -A, B, and C are discussed toward the end of this paper. The following paper (IV) is concerned with the characteristics of the genetic forms of β -casein.

MATERIALS AND METHODS

Sources of α_s -casein variants. α_s -Caseins A, B, and C each obtained from milk of individual cows known to produce only one type of α_s -casein. α_s -B casein was obtained from individual Holstein cows, while α_s -C was obtained from Guernsey cows. α_s -A casein, the most difficult to locate (5), was obtained from individual Holstein cows. In no instances were casein preparations from different cows pooled.

Preparation of α_s -caseins. Casein was precipitated from fresh skim milk at pH 4.6-4.8, 25°C with 1 N HCl. The precipitate was washed four to five times with distilled water, with the pH maintained at 4.6-4.8, redissolved at pH 7.5, reprecipitated, and washed. Two hundred grams of whole acid casein (~100 g dry weight) was dissolved in two liters of 6.6 M urea and diluted to 3.3 M urea at pH 4.6-4.8. The precipitate (α -casein complex), obtained by centrifugation, was redissolved in 6.6 M urea and again diluted to 3.3 M urea. After removing the urea by six to eight successive washings in a Waring blender, the α -casein complex was dissolved in about 800 ml of distilled water at pH 7.2, chilled to 2-4°C, and

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¹ Eastern Utilization Research and Development Division, Agricultural Research Service, USDA.

4.0 M CaCl_2 added slowly, with rapid stirring, to 0.40 M. After warming to 20°C and stirring for 30 min, the precipitate (crude α_s) was obtained by centrifugation at $2,000 \times g$ for 15 min and dissolved in 5.0 urea. (At this stage of the preparation α_s -B and α_s -C quantitatively precipitated upon centrifugation. However, with α_s -casein A it was necessary to warm the mixture to 30°C before maximum precipitation occurred.) Upon dilution of the solution to 1 M urea at pH 4.6, the bulk of the calcium remained in the supernatant. The remainder of the calcium was removed by thrice dissolving the precipitate in distilled water at pH 7.5, followed by acid precipitation. After repeating the calcium fractionation step, followed by the removal of calcium as outlined, the crude α_s -casein was dissolved at pH 7.0 in 500 ml distilled water, an equal volume of absolute ethanol added, and 2 M ammonium acetate added dropwise with stirring until a definite precipitate formed (21). The precipitate was removed by centrifugation, discarded, and the supernatant diluted to 10% ethanol at pH 4.6. The gummy precipitate was dissolved at pH 7.5, in a minimum volume of water, dialyzed, concentrated, and lyophilized. Yields of α_s -B and α_s -C caseins were approximately 20 g, whereas yields of α_s -A approximated 12-15 g.

DEAE-cellulose-urea chromatography. DEAE-cellulose, obtained from Distillation Products Industries,² with an exchange capacity of about 0.90 meq/g, was slurried with distilled water and adjusted to about 0.5 N NaOH. After settling for 0.5 hr, the fines were decanted and the slurry filtered on a Büchner funnel. The filter cake was washed with several volumes of water, filtered, slurried, and the pH adjusted to 7.0 with 1 N HCl. The slurry was filtered, the filter cake added to 0.01 M imidazole-HCl buffer at pH 7.0 (9), and again filtered. After slurrying and filtering five to six times with the imidazole-HCl buffer, the DEAE-cellulose was allowed to equilibrate overnight, again filtered, and additional volumes of buffer added.

The columns (2 by 30 cm) were poured to a depth of 20 cm under a pressure of 6.5 psi with several slurries of the DEAE-cellulose. A liquid head was maintained to prevent drying of the column. The protein, usually 500-800 mg, was dissolved in a minimum volume (about 25 ml) of 3.3 M urea, 0.01 M imidazole-HCl buffer. It was applied to the column and the

sides of the column were washed three times with 5-ml volumes of urea buffer. A 2-cm liquid head was maintained.

Elution chromatography was carried out using a Technicon Autograd mixing apparatus. For α_s -casein we have found it expedient to begin the NaCl gradient at 0.10 M NaCl. Thus, the elution schedule was as follows: nine cylinders, each containing 150 ml of 3.3 M urea in 0.01 M imidazole-HCl buffer, were used and were 0.10, 0.13, 0.15, 0.17, 0.19, 0.21, 0.23, 0.25, and 0.27 M NaCl, respectively. The rate of elution was about 80 ml/hr, and 10-ml aliquots were collected. Elution of protein was monitored at 280 m μ with a Canaleco Universal F UV Analyzer. For quantitation, eluted protein was read at 280 m μ in a Beckman DU spectrophotometer. The eluted protein was collected and dialyzed urea-free at 4°C. Ordinarily, the complete removal of urea took five to six days. The dialyzed protein was concentrated and lyophilized unless otherwise stated.

Preparation of samples for analyses. Use of statically charged lyophilized proteins makes them difficult to handle. For chemical analyses the lyophilized chromatographed α_s -caseins were dissolved at pH 7.0, precipitated at pH 4.6, and washed five to six times with distilled water. The residue was solvent-dried by three to four extractions each with absolute ethyl alcohol, acetone, and ether. The dried protein was finely ground in a Wiley mill. The homogeneous powders were allowed to equilibrate three days under constant humidity and temperature prior to analyses. The moisture content of the powders ranged from 6-8%.

Phosphorus, nitrogen, and absorptivities. Phosphorus analyses were run in duplicate on 10-mg samples by the method of Sumner (11). Nitrogen analyses were run in triplicate by the Kjeldahl method. Absorptivities (A 1%) were determined at 280 m μ in a 1-cm cell path with protein solutions containing 1 mg/ml, corrected for moisture. All values are reported on a moisture-free basis.

Stabilization tests. The ability of κ -casein to stabilize α_s -caseins A, B, and C against precipitation by calcium ions was studied by the method of Zittle (20).

Zonal electrophoresis. Starch-gel-urea electrophoresis (SGE) was performed by the method of Wake and Baldwin (18), at pH 8.6, 7.0 M urea. Relative mobility measurements were made using the zone described by Wake and Baldwin as 1.00 as standard. Polyacrylamide-gel electrophoresis (PAE), at pH 9.1-9.3, 4.5 M urea, as previously described (13) was the principal electrophoretic method used. In

²It is not implied the U. S. Department of Agriculture recommends the above company or its products to the possible exclusion of others in the same business.

addition to the advantages of this method previously cited (13), the method is also sensitive to small amounts of contaminating protein. SGE zone 1.00 was identified and similarly assigned for PAE, from which relative mobilities of the proteins were designated.

RESULTS AND DISCUSSION

The elution characteristics of α_s -caseins A, B, and C from DEAE-cellulose, pH 7.0, 3.3 M urea, are presented in Figures 1, 2, and 3. The diagonal broken line shown in Figure 1 represents the salt gradient used in the chromatography of the three α_s -casein variants. The linear gradient is identical in Figures 2 and 3. Several observations can be made from these diagrams. First, α_s -casein A (Figure 1) differs from the two other variants in exhibiting a bimodal elution pattern on chromatography. Rechromatography of either the main peak (vertical, broken lines) or the shoulder yields bimodal elution behavior in each. Such

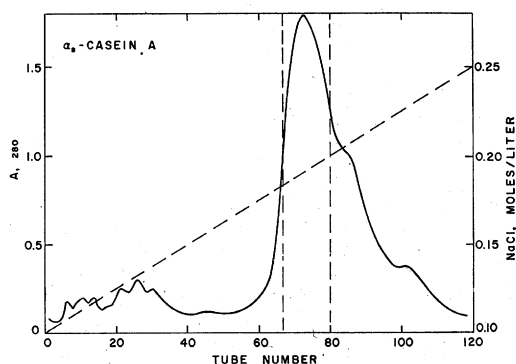


FIG. 1. Elution diagram (absorbance, A, 280 m μ , versus tube number) of α_s -casein A from DEAE-cellulose, pH 7.0, imidazole-HCl urea buffer, 540 mg protein charge.

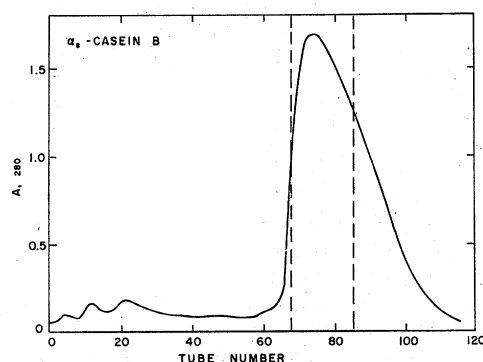


FIG. 2. Elution diagram (absorbance, A, 280 m μ , versus tube number) of α_s -casein B from DEAE-cellulose, pH 7.0, imidazole-HCl urea buffer, 540 mg protein charge.

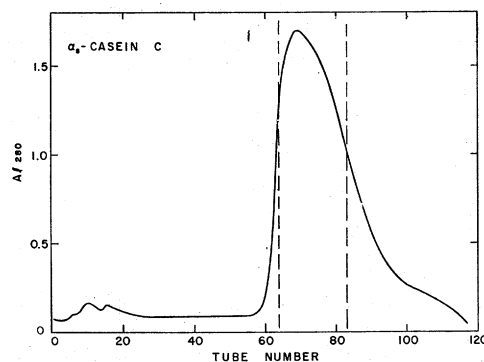


FIG. 3. Elution diagram (absorbance, A, 280 m μ , versus tube number) of α_s -casein C from DEAE-cellulose, pH 7.0, imidazole-HCl urea buffer, 540 mg protein charge.

behavior is suggestive of incomplete dissociation of α_s -A in 3.3 M urea, rather than inhomogeneity of the fraction, for PAE of either the main peak or shoulder gives zones of identical mobility. Second, α_s -A is more contaminated (Table 1) than either α_s -B or α_s -C, although preparative methods were the same. Third, α_s -B (Figure 2) and α_s -C (Figure 3) are surprisingly homogeneous prior to column chromatography; there appears to be only 10% impurity. Fourth, the position of elution (about 0.20 M NaCl) of all three variants suggests that chromatographic separation of heterozygous α_s -caseins (AB, AC, and BC), under the conditions described in this paper, is virtually impossible. This supposition is borne out by fruitless attempts to separate any of the above heterozygotes. Therefore, homozygous-typed caseins appear as the most likely starting source of these genetic variants.

Prior to analyses, fractions (indicated by the

TABLE 1

Chromatographic properties of α_s -casein variants eluted from DEAE-cellulose columns (2 \times 20 cm) at pH 7.0, imidazole-HCl urea buffer, and relative mobilities by zonal electrophoresis

Variant	Position of elution moles NaCl/liter	Per cent protein in major peak	Relative mobility	
			SGE	PAE
α_s -A	0.20	82.0 ^a	1.18 ^b	1.22 ^c
α_s -B	0.20	90.4	1.10	1.13
α_s -C	0.20	90.5	1.07	1.10

^a Including the chromatographic shoulder.

^b Using Wake and Baldwin's zone 1.00 as reference.

^c Using the corresponding SGE zone 1.00 (applied to) as reference for PAE.

vertical broken lines in Figures 1, 2, and 3) were collected, dialyzed, lyophilized, and re-chromatographed. Only traces of impurities were then observed. Figures 4 (Patterns 1, 2, and 3) and 5 (Patterns 2, 3, and 4) show the purity of twice-chromatographed α_s -caseins A, B, and C, respectively. Only traces of impurity are disclosed by PAE and SGE. Prior to column chromatography, only small amounts of impurity were observed on PAE. Although gel

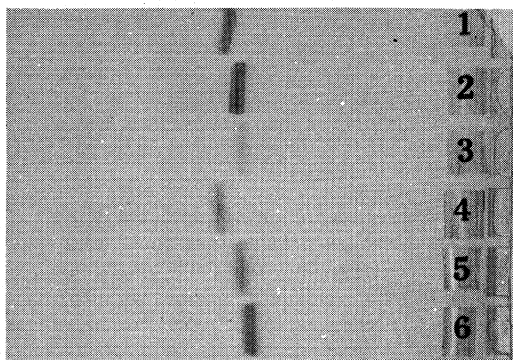


FIG. 4. Polyacrylamide-gel electrophoresis at pH 9.1-9.3, Tris- Na_2EDTA -borate buffer, 4.5 M urea. Patterns 1, 2, and 3 show twice-chromatographed α_s -A, B, and C, respectively. Patterns 4, 5, and 6 show the variants prior to chromatography.

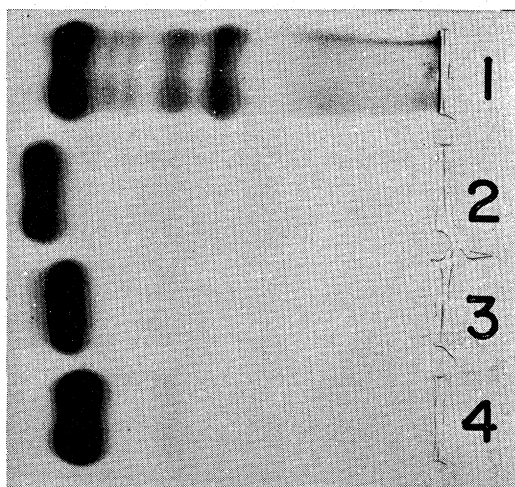


FIG. 5. Starch-gel electrophoresis patterns at pH 8.6, Tris-citrate buffer, 7.0 M urea, of 1. α_s -B, β -A typed whole casein, 2, 3, and 4 α_s -A, B, and C, respectively.

electrophoresis is a very discriminating method for determination of the purity of casein components, column chromatography revealed the presence of more impurity than did the electrophoretic methods. This may be explained by the fact that some caseins bind less dye than

others. Two-dimensional electrophoresis of the purified genetic variants does not disclose any further heterogeneity.

Since relative mobility of casein components on SGE or PAE is especially useful in their identification, such values are presented in Table 1. Although the mobilities on SGE and PAE differ, the magnitude of the differences is about the same. For example, on SGE the differences between α_s -A and B and α_s -B and C are 0.08 and 0.03, respectively, whereas on PAE they are 0.09 and 0.03. The relative mobilities depend, of course, upon gel strength and the buffer used.

Criteria for α_s -caseins. The ability of the genetic variants of α_s -casein to be stabilized by κ -casein in the presence of 0.01 M CaCl_2 is

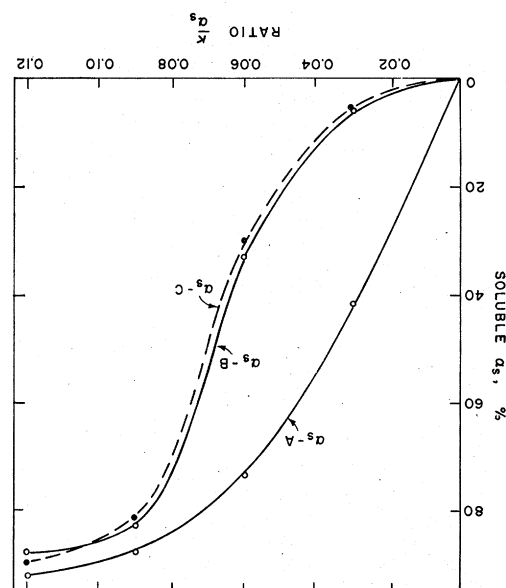


FIG. 6. Stabilization curves for α_s -caseins A, B, and C obtained by the method of Zittle (20).

shown in figure 6. Several conclusions can be drawn from this figure: (a) All three variants are quantitatively precipitated by 0.01 M CaCl_2 ; (b) α_s -B and α_s -C are stabilized at the same weight ratio of κ/α_s ; (c) α_s -A casein shows the most unusual behavior in that it is stabilized at a lower level of κ -casein and a greater percentage of the protein is stabilized against precipitation with Ca^{++} . Some analogy may be drawn between this observation and the unusual preparative characteristic of the α_s -A/ κ complex, which is completely soluble at 4°C in 0.4 M CaCl_2 and requires a temperature of 30°C for complete precipitation of the α_s -casein. The analogy is that in both instances α_s -A

strongly interacts with κ -casein, producing a micelle of greater stability than with α_s -B or α_s -C. Similarly, α_s -AB and α_s -AC are completely soluble at 4°C in 0.40 M CaCl_2 . Nevertheless, α_s -casein A, B, and C all satisfy a criterion of α_s -caseins (19), in that they form micelles in the presence of Ca^{++} . The formation of ultracentrifugal α_s - κ casein complexes, by the three α_s variants, is yet to be ascertained. Because α_s - κ complexes are clottable with rennin, in the presence of divalent ions, another criterion of an α_s -casein is satisfied.

Physical and chemical properties. Absorptivities (A 1%) are 10.10, 10.05, and 10.03 for α_s -A, B, and C, respectively, suggesting larger quantities of tryptophan or tyrosine in α_s -A. Waugh et al. (19) reported a value of 10.1 for a mixture of α_s -caseins termed $\alpha_{s1.2}$. A recent value (10.2) by Zittle and Custer (21) for an α_s -casein (reported as predominantly B) is in fair agreement with our results.

Comparisons of calcium-sensitive α_s -caseins have been made largely on the basis of chemical analyses. The value of such comparisons is debatable, in view of the suspected heterogeneity and over-all purity of the components. On pure fractions, however, such information becomes valuable. α_s -A, B, and C contain 15.10, 15.34, and 15.40% nitrogen, respectively, which are of the same order as the 15.10 value of Zittle and Custer (21). Values of 14.10% for α_1 -casein (8), 14.5% for $\alpha_{1.2}$ (19), and 14.0% for α -caseins 1.07 and 1.10 (10) are much lower than for α_s -caseins reported in this study. The reason for these discrepancies is unclear, but could be explained on the basis of (a) loss of amide nitrogen, (b) impurities of low nitrogen content, or (c) inorganic contamination. Since whole α -casein has been reported to contain 15.57% nitrogen, and if κ -casein with a nitrogen content of 14.5% comprises about 25% of the complex, then the differences in nitrogen could not be reconciled by the presence of κ -casein as a contaminant.

All three α_s -caseins contain 1.01% phosphorus (Table 2) or 9 g atoms per mole of 27,500 molecular weight (19). Zittle and Custer (21) and Waugh et al. (19) report almost identical values. Schmidt and Payens (10)

report a phosphorus value of 1.12% or about 6 g atoms per mole of 16,500 molecular weight, or ten per 27,500. McMeekin et al. (8) reported 0.85% phosphorus for α_1 -casein, and Long et al. (6) 1.18% for α_R -casein. The P/N ratios (16) are almost identical for α_s -A, B, and C, and each variant is free of carbohydrate, cystine, and cysteine (17).

Because PAE and SGE are the most discriminating methods available for the study of casein components, some comparisons of behavior and relative mobilities can be made among reported proteins. Schmidt and Payens (10), employing SGE according to Wake and Baldwin (18), designate their α -caseins as zones 1.07 and 1.10 and believe these two zones correspond to α_s -BC of Thompson et al. (15). Recently, Thompson et al. (13) reported values of 1.07 for α_s -B and 1.10 for α_s -C, confirming the suggestion of Schmidt and Payens (10). Waugh et al. (19) reported $\alpha_{s1.2}$ to be in the SGE zone region of 1.01-1.14, although precise positions were not reported. That Schmidt and Payens and Waugh et al. did not isolate α_s -A seems certain from its relative mobility on SGE (1.18) and from its low frequency in milks of Holstein cows (5). α_s -A has not been found in the milks of any other breed.

The temptation to speculate what $\alpha_{s1.2}$ actually are is great. Waugh et al. (19) reported their source of milk to be individual Guernsey cows, a breed with gene frequencies for α_s -B of 0.70 and α_s -C of 0.30. Thus, the likelihood of procuring an α_s -B/C heterozygote is good. Unfortunately, they failed to recognize the earlier studies of Aschaffenburg (1) on genetic variation in β -casein or the work of Thompson et al. (14, 15) on α_s -caseins.

Further comparisons of α_s -A, B, and C with other calcium-sensitive α -caseins may be made on the basis of end group analyses, each of the three α_s -variants possessing N-terminal arginine and C-terminal tryptophan (4). These studies have virtually excluded the possibility of other end groups, and on the basis of the amount of C-terminal tryptophan released, a molecular weight of about 30,000 is calculated for each variant, assuming a single polypeptide chain. Waugh et al. (19) calculated a

value of 27,000-27,500 from tryptophan released, but also reported the presence of leucine and tyrosine as possible end groups. Leucine is penultimate (4) in α_s -A, B, and C. Other researchers (7, 10) have also shown arginine to be N-terminal in calcium-sensitive α -caseins.

From the foregoing discussion on the properties of calcium-sensitive α -caseins some conclusions can be drawn regarding similarities among those caseins. First, $\alpha_{s1,2}$ (19) and α -caseins 1.07 and 1.10 (10) probably correspond to the genetic variants α_s -B and α_s -C. A difference in concentration of one component compared to the other would be expected when pooled milk is used as a protein source. Second, α_R (6) and α_1 (8) have not been adequately compared with recent preparations of α_s -caseins. Considering the source (α -casein complex) of these components, however, it seems reasonable to assume that they differ only in purity from the components previously discussed in this paper. Third, terminology of the caseins has become extremely confusing, thus warranting a nomenclature which can be applied to identical fractions (except for purity) and include others yet to be discovered and named.

Nomenclature of α_s -caseins. α_s -Caseins A, B, and C qualify as α_s -caseins by definition (19). Furthermore, each is a single component under the electrophoretic conditions reported. We feel that the term, α_s -casein, is both descriptive and succinct and should supersede in use similarly named fractions. The term $\alpha_{s1,2}$, however, is unnecessary, because it is fairly certain that these two components represent α_s -BC as do α -caseins 1.07 and 1.10. The discovery of α_s -casein variants does, therefore, simplify rather than confuse the nomenclature. Some concern exists over the presence and naming of additional α_s -like caseins (19). SGE zones 1.04, 1.00, and 0.86 appear to qualify as α_s -caseins (unpublished results). Therefore, the nomenclature must indicate this.

The suggestion of Waugh et al. (19) that other α_s -caseins be termed α_{s2} , α_{s3} , etc., has considerable merit. It is unfortunate from the nomenclature standpoint that $\alpha_{s1,2}$ was a two-component system. The use of relative mobility, as applied to α -casein 1.07 and 1.10, for example, was suggested by Wake and Baldwin (18) as an alternate method of naming and, therefore, is useful for identification of casein components.

We feel that the term α_{s1} should be applied to the principal α_s -casein fraction formerly termed α_s -A, B, and C, $\alpha_{s1,2}$, and α -caseins 1.07

and 1.10, and that henceforth the genetic variants of α_{s1} be termed α_{s1} -A, α_{s1} -B, and α_{s1} -C. For precise identification on SGE, for example, these caseins would be termed: α_{s1} -A (1.18); α_{s1} -B (1.10); α_{s1} -C (1.07).

The proposed scheme is sufficiently flexible to include other possible variants of the α_{s1} series. For example, if a variant controlled by an allele at the α_{s1} locus is discovered which has a relative mobility of 1.14, it would be termed α_{s1} -D (1.14). Figure 7 shows the rela-

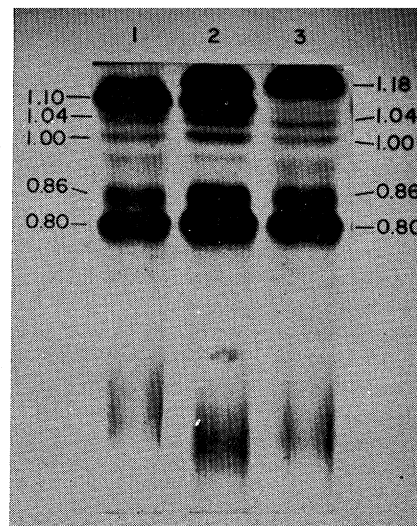


FIG. 7. Starch-gel electrophoresis patterns showing a portion of the zone numbering system of Wake and Baldwin (18). Pattern 1 is α_s -B typed whole casein; 2, α_s -AB; and 3, α_s -A.

tive mobility of α_{s1} -A (1.18) and α_{s1} -B (1.10). The isolation characterization of other α_s -like caseins, for example, zones 1.04, 1.00, and 0.86 (Figure 7) would be termed α_{s2} (1.04), α_{s3} (1.00), and α_{s4} (0.86) in order of characterization, which is consistent with the proposal of Waugh et al. (19).

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